

Bioremediation of cyanide-containing wastes

The potential of systems and synthetic biology for cleaning up the toxic leftovers from mining

María Dolores Roldán^{1,*}, Alfonso Olaya-Abril¹, Lara P Sáez¹, Purificación Cabello², Víctor M Luque-Almagro¹ & Conrado Moreno-Vivián¹

Cyanide is a highly toxic chemical produced in large amounts by the mining and jewellery industries, steel manufacturing, coal coking, food processing and chemical synthesis (Luque-Almagro *et al.*, 2011). The mining industry uses so-called cyanide leaching to extract gold and other precious metals from ores, which leaves large amounts of cyanide-containing liquid wastes with arsenic, mercury, lead, copper, zinc and sulphuric acid as cocontaminants.

Although these techniques are very efficient, they still produce about one million tonnes of toxic wastewaters each year, which are usually stored in artificial ponds that are prone to leaching or dam breaks and pose a major threat to the environment and human health (Luque-Almagro *et al.*, 2016). In 2000, a dam burst in Baia Mare, Romania, caused one of the worst environmental disasters in Europe. Liquid waste from a gold mining operation containing about 100 tonnes of cyanide spilled into the Somes River and eventually reached the Danube, killing up to 80% of wildlife in the affected areas. A more recent spill was caused by a blast furnace at Burns Harbor, IN, USA, which released 2,400 kg of ammonia and 260 kg of cyanide at concentrations more than 1,000 times over the legal limit into Calumet River and Lake Michigan, severely affecting wildlife. Notwithstanding the enormous damage such major spills cause, industrial activities that continuously release small amounts of waste are similarly dangerous for human and environmental health.

The European Parliament, as part of its General Union Environment Action Programme, has called for a ban on cyanide in mining activities to protect water resources and ecosystems against pollution. Although several EU member states have joined this initiative, there is still no binding legislation. Similarly, there are no general laws in the USA to prevent cyanide spills, and former administration even authorized the use of cyanide for control predators in agriculture.

Cyanides

The cyano group ($-C\equiv N$) is naturally present in many natural compounds. At neutral and acidic pH, hydrogen cyanide (HCN) is the predominant form, while the anion (CN^-) prevails at alkaline pH. Other forms of cyanide are metal–cyanide complexes, which include weak complexes (Ni, Cu, or Zn) and very strong ones (Fe or Co). Cyanate (OCN^-) and thiocyanate (SCN^-) are oxidized cyanide derivatives. Finally, organic cyanides comprise cyanohydrins (2-hydroxynitriles), aliphatic, aromatic and aryl-aliphatic nitriles, and different cyano-derivatives of lipids, carbohydrates and other biological compounds. The toxicity of cyano-derivatives depends on their capacity to release free cyanide. The most toxic forms are HCN and CN^- , while the oxidized forms cyanate or thiocyanate show an intermediate toxicity and nitriles usually have low toxicity. The free cyanide binds to the metal cofactors of key proteins, such as haemoglobin and cytochrome *c* oxidase,

causing loss of function. The transport of oxygen and carbon dioxide in the body and cellular respiration are particularly sensitive to cyanide, which causes death by asphyxiation.

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Legislators and affected communities increasingly require more strict control to prevent leakages or take to account unscrupulous companies that cut short on safety measures to prevent cyanide spills. Another key solution to the huge amount of cyanide waste is the search for new technologies to clean up with contaminated areas. Several physical–chemical processes, such as ozonization, alkaline chlorination, hydrogen peroxide oxidation and other methods, can be applied to detoxify cyanide-containing wastes, but biodegradation is the most eco-friendly and sustainable alternative. In this sense, synthetic biology provides tools for designing optimized microorganisms to remove toxic contaminants that could contribute to any solutions to deal with cyanide pollution. Systems biology, along with omics technologies, such as genomics, transcriptomics, proteomics and metabolomics, has already generated a huge amount of data on microbial organisms to better understand the

1 Departamento de Bioquímica y Biología Molecular, Edificio Severo Ochoa, Universidad de Córdoba, Córdoba, Spain

2 Departamento de Botánica, Ecología y Fisiología Vegetal, Edificio Celestino Mutis, Universidad de Córdoba, Córdoba, Spain

*Corresponding author. Tel: +34 957218588; E-mail: bb2rorum@uco.es

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metabolic pathways needed for biological detoxification (Kallergi *et al.*, 2021). In the case of cyanide-containing wastes, the most thoroughly studied microorganism able to detoxify elevated concentrations of cyanide of up to 12 mM is the alkaliphilic bacterium *Pseudomonas pseudoalcaligenes* CECT5344, a strain with great biotechnological potential, which is also able to tolerate elevated concentrations of metals (Ibáñez *et al.*, 2017).

An overview of microbial degradation of cyanide

Generally, any cyanotrophic bacteria and fungi that are able to use cyanide and its derivatives as nitrogen source for growth

may be used for bioremediation processes. These organisms usually have metabolic pathways for cyanide degradation along with mechanisms to prevent cyanide poisoning like the cyanide-insensitive alternative oxidase (Cio) (Fig 1). Depending on the organisms, inorganic cyanide is degraded mainly by oxidative, substitution/transfer or hydrolytic reactions (Luque-Almagro *et al.*, 2018). Cyanide oxidation to ammonia and carbon dioxide occurs directly or in two steps through the formation of cyanate. Substitution/transfer reactions first generate less toxic intermediates, such as thiocyanate or 3-cyanoalanine (3CNA), which are further degraded to ammonia. Hydrolytic reactions are the main mechanism to degrade both

inorganic (CN^-) and organic (R-CN) cyanides. Cyanidase (cyanide dihydratase) directly converts inorganic cyanide into formic acid and ammonia, whereas cyanide hydratase transforms cyanide into formamide, which is subsequently degraded to formic acid and ammonia. Cyanide hydratases are usually fungal enzymes, whereas cyanidases are found in several bacterial species. Nitriles are similarly hydrolysed to ammonia and the corresponding carboxylic acid either directly by a nitrilase, or via an amide by a nitrile hydratase (Fig 1A).

Biochemical and omics studies of *P. pseudoalcaligenes* CECT5344 have demonstrated that nitrilase NitC is essential for the assimilation of both inorganic and organic

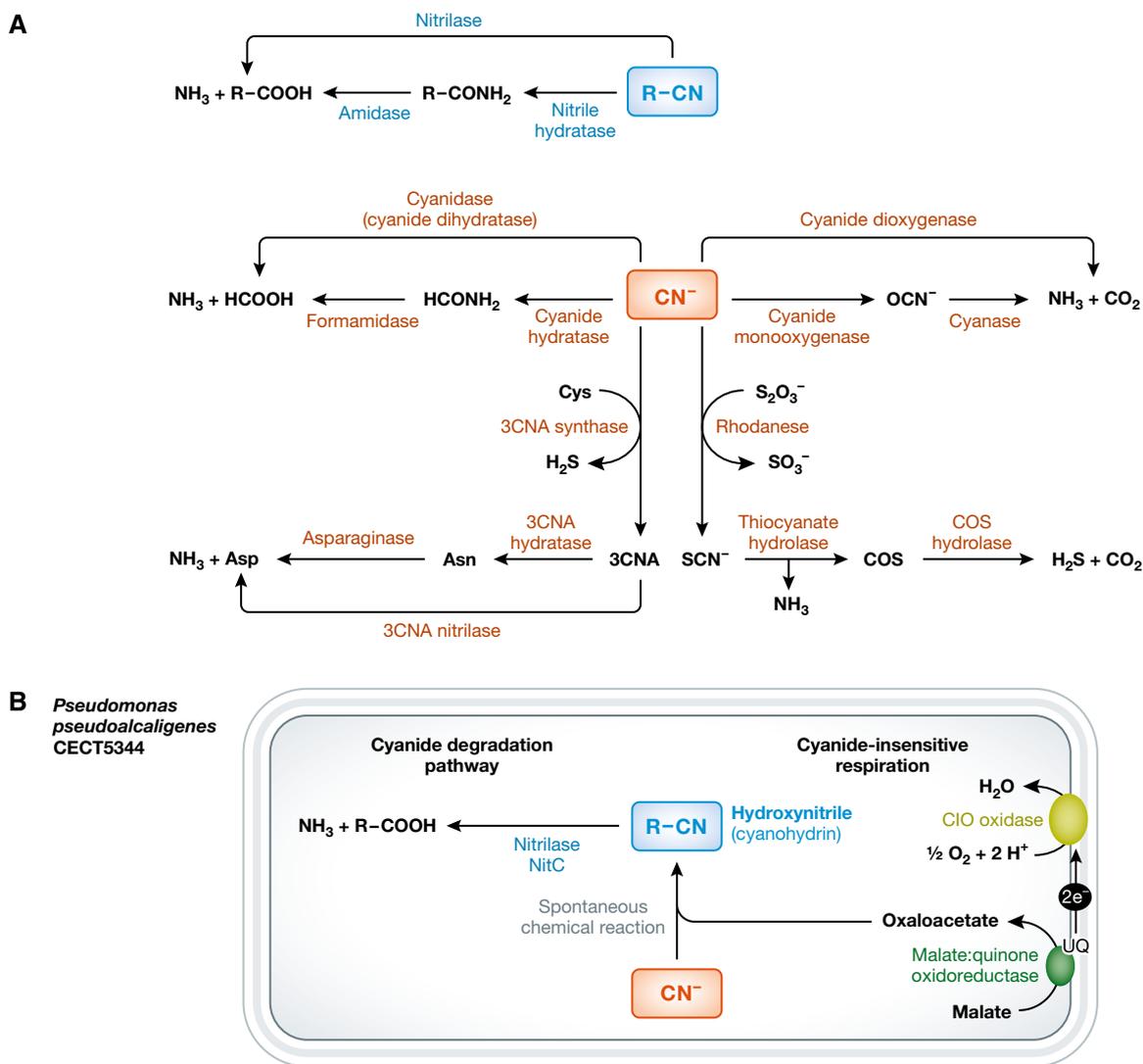


Figure 1. Enzymatic cyanide degradation pathways (A) and the links between cyanide assimilation and resistance in *P. pseudoalcaligenes* CECT5344 (B).

cyanides (Fig 1B) and that the cyanide degradation pathway and cyanide-resistant respiration are linked. The *nit1C* gene cluster codes for the nitrilase NitC, which is induced by cyanide (Estepa *et al*, 2012), while the alternative oxidase encoded by the *cio* gene cluster enables cyanide-insensitive respiration. The Cio oxidase receives electrons from ubiquinone, which in turn is reduced by a malate:quinone oxidoreductase that oxidizes malate to oxaloacetate. The connection between cyanide assimilation and tolerance arises from the fact that inorganic cyanide reacts with oxaloacetate generated during the respiratory electron transfer to form a 2-hydroxynitrile as the substrate for the nitrilase, which releases ammonium as a nitrogen source for growth (Fig 1B). NitC may also hydrolyse cyanohydrins formed by reactions of cyanide with other ketoacids such as oxoglutarate or pyruvate (Estepa *et al*, 2012). The genome of *P. pseudoalcaligenes* CECT5344 actually contains four nitrilase genes, which allows it to assimilate different nitriles. The NitC-dependent pathway has been also identified in *Pseudomonas fluorescens* NCIMB 11764 (Jones *et al*, 2018) and many other bacteria from different taxonomic groups, suggesting a key role of this enzyme in bacterial cyanide degradation.

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Cyanomics: a holistic view of the cyanide biodegradative process

Omics technologies provide a holistic view of biological processes as they generate information on genes (genomics), mRNA and sRNA (transcriptomics), proteins (proteomics) and metabolites (metabolomics). During the past twenty years, these technologies have made remarkable advances in terms of improved performance, sensitivity and reproducibility. Omics techniques were initially performed with isolated bacterial strains, but the impossibility to cultivate most autochthonous microorganisms in synthetic media (about 99% are non-cultivable) has inspired culture-

independent technologies, called metaomics, which allow to study microbial communities in natural ecosystems for their genetic potential (metagenomics) and their functional capacity (metatranscriptomics and metaproteomics). The huge accumulation of sequences and other information thus generated required a parallel evolution of bioinformatic tools to store and process this “big data” (Fig 2).

Environmental omics emerged as a research field to use these technologies for a better understanding of the effect of environmental pollution on individual organisms and whole ecosystems (Malla *et al*, 2018). This knowledge has potentiated the development of environmental biotechnology, specifically in biodegradation, bioremediation and monitoring of chemical pollutants. Cyanide pollution and biodegradation have specifically been addressed as so-called cyanomics (Luque-Almagro *et al*, 2016).

“This genome-based prospection for cyanotrophy [...] could be a successful tool for identifying microorganisms suitable for bioremediation ...”

Pseudomonas pseudoalcaligenes CECT5344 was the first cyanide-assimilating bacterium for which the whole genome was sequenced (Luque-Almagro *et al*, 2013) and its methylome was also established (Wibberg *et al*, 2016). Transcriptomics and proteomics helped to identify further genes/proteins involved in cyanide and metal resistance and regulatory roles (Luque-Almagro *et al*, 2015; Ibáñez *et al*, 2017), as well as putative regulatory small RNAs (Olaya-Abril *et al*, 2019). However, the availability of genomes from cyanotrophic bacteria is still limited to *Pseudomonas* strains *P. pseudoalcaligenes* CECT5344, *P. fluorescens* NCIMB11764 and *P. monteilii* BCN3. Nonetheless, several other non-cyanotrophic bacteria were found to harbour genes for cyanide degradation. Sequencing of a high number of cyanide-assimilating bacteria and further comparative genome analysis among cyanotrophic and non-cyanotrophic bacteria could thus help to identify further genes involved in cyanide degradation as predictors for screening other bacterial genomes. This genome-based prospection for cyanotrophy, also considering genes involved in resistance to heavy metals and other toxic chemicals, could be a successful tool for identifying microorganisms suitable

for bioremediation of specific cyanide-containing industrial wastewaters.

Other omics technologies have been poorly applied to cyanide biodegradation; in fact, transcriptomic and proteomic studies have so far been carried out only in *P. pseudoalcaligenes* CECT5344 to link the *nit1C* gene cluster to cyanide assimilation and the *cio* gene cluster to cyanide-insensitive respiration (Estepa *et al*, 2012; Luque-Almagro *et al*, 2015; Ibáñez *et al*, 2017). A homologous *nit1C* gene cluster was also identified in *P. fluorescens* NCIM11764 (Jones *et al*, 2018), and it may be present in more than 2,700 bacteria with the conserved protein domain family *nitrile_sl0784*.

“Cyanomic studies will provide useful tools to design, construct and optimize the cyanide biodegradation process that can be applied to bioremediation ...”

Metagenomic approaches have revealed the structure of microbial communities from cyanide-containing environments, such as coking and gold ore wastewaters (Wang *et al*, 2015). Similar metagenomic studies based on function-driven screening have been applied to identify nitrilases and other degradative enzymes with novel capabilities (Sonbol *et al*, 2016). Future metatranscriptomics and metaproteomics approaches could functionally assess microbial communities from cyanide-contaminated sites. This knowledge would be relevant for future engineering of niches within mixed-culture biotechnological processes, which could be applied for the restoration of ecosystems devastated by cyanide and other pollutants.

Synthetic biology applied to cyanide-containing waste detoxification

Cyanomic studies will provide useful tools to design, construct and optimize the cyanide biodegradation process that can be applied to bioremediation of cyanide-containing wastes *in vivo* or *ex vivo* (Fig 2). The first step is to acquire global knowledge of the biodegradative process in a model microorganism (Rylott & Bruce, 2020). However, high-throughput analyses are not enough to characterize the interactions and behaviour of biological entities. In

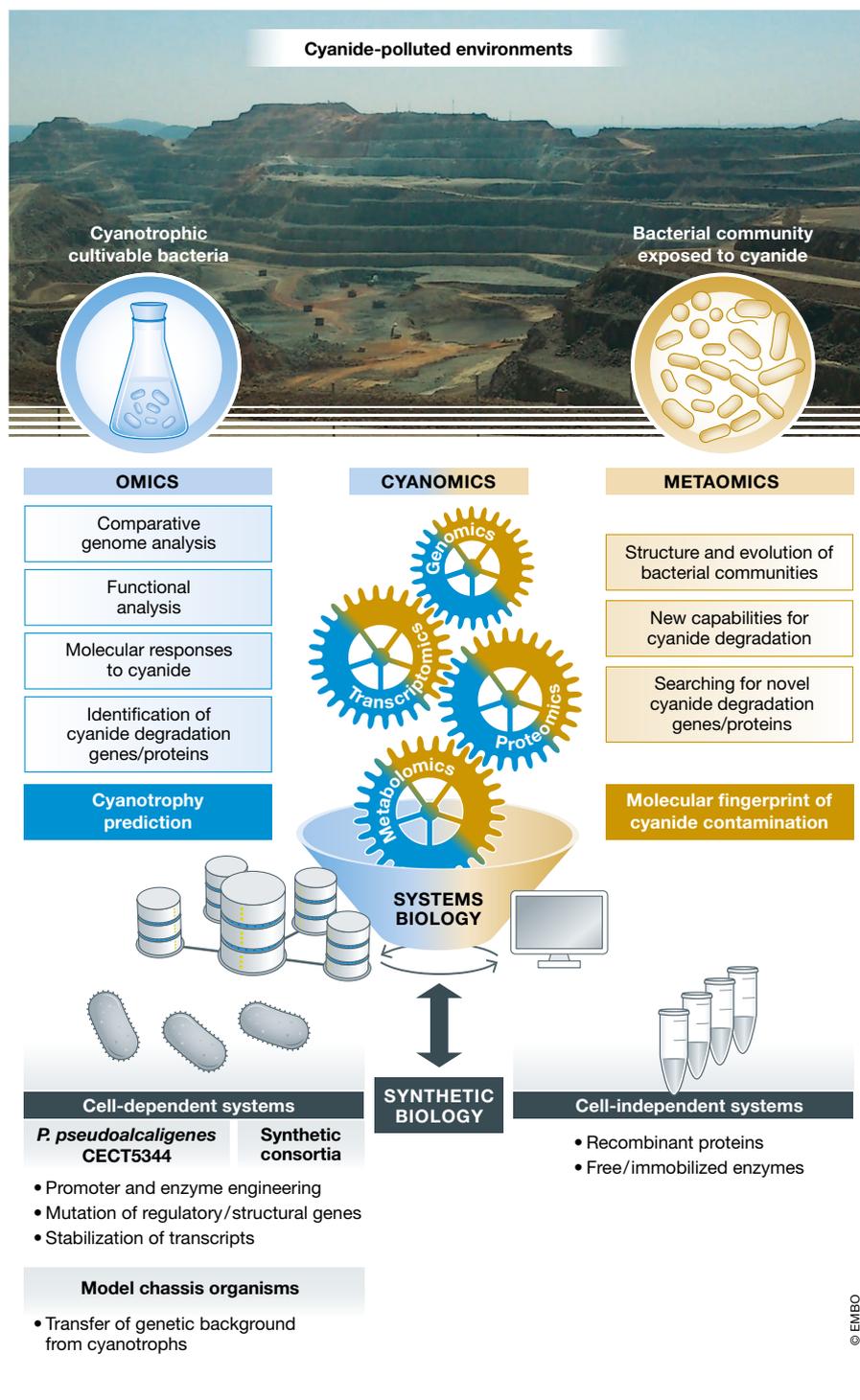


Figure 2. Use of synthetic biology for bacterial cyanide bioremediation.

this sense, the development of new computational tools and algorithms, the functional validation of the results obtained from omics analyses and more data are of great help.

However, in some cases the information available may be scarce. This is especially

relevant for high-throughput untargeted metabolomic studies applied to cyanide metabolism, a compound that can react easily with many different biomolecules, thus generating derivatives that are difficult to identify. In addition, to adequately

execute good experimental designs, it is necessary to set up appropriate controls and sufficient biological and technical replicas. Once robust and validated data are obtained, the metabolic pathways involved in cyanide resistance and assimilation/detoxification can be inferred, as well as changes caused by the adjustment of metabolic fluxes in the presence of cyanide and other biomolecules.

Pseudomonas pseudoalcaligenes CECT5344 could thus become a chassis organism for synthetic biology to fully optimize the cyanide bioremediation process via design–build–test–learn (DBTL) cycle (Fig 2). Without introducing new exogenous functions, development could concentrate on the metabolic bottlenecks and use site-directed mutagenesis of regulatory genes and/or specific promoters to increase the stability of transcripts, or to enhance the activity of enzymes such as the nitrilase NitC through protein engineering.

Another approach is to add new functionalities to the CECT5344 strain. Available nucleotide databases are one source of putative gene sequences, but to discover new features, proteogenomics from environments contaminated with cyanide may be needed. Using CECT5344 as a microbial chassis for other genes involved in alternative cyanide biodegradation routes, such as those coding for cyanidase, cyanide hydratase and/or nitrilases, could improve its cyanide resistance/detoxification pathways. In this sense, siderophore production may be of interest to compensate cyanide-driven iron starvation, while enhancement of antioxidant defences such as superoxide dismutase or peroxidases may alleviate the oxidative stress caused by cyanide and metals.

A third strategy would involve the transfer of the CECT5344 genetic background for cyanide biodegradation, together with other traits to a well-described model bacterial strain (*E. coli*, *P. putida*). This could help to improve growth rates for various environmental factors such temperature, oxygenation, salt composition or pH. It may be more difficult to achieve, however, than improving the CECT5344 strain because the cyanide resistance/detoxification process is extremely complex. Stress generated by the presence of cyanide and changes induced in the general physiology require compensation, which could be a difficult challenge given the number and diversity of the mechanisms involved.

Finally, the use of new microorganisms isolated from contaminated environments as chassis for synthetic biology would even be

more challenging as the genetic background and physiology are not well characterized and growth rates and gene editing techniques would require considerable optimization. In any case, it would require a lot of further work to reduce the stress exerted by cyanogenic compounds on the host cell's general physiology, along with fine-tuning the expression levels of assembled pathways, and controlling interactions between exogenous and endogenous metabolic pathways and compounds.

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A different option is establishing natural or synthetic microbial consortia, using bacteria and/or fungi with different cyanide biodegradation pathways either from natural bioaugmented samples or from cyanotrophic strains. A key point is that these microorganisms should have similar growth rates in the given medium or environment to maintain the overall stability of consortium. This requires sensitive tools to analyse the stability of consortia and taking other aspects such as quorum sensing into consideration. Finally, whole-cell extracts or purified proteins could be used to assemble *in vitro*, cell-free systems to convert cyanide and cyanide derivatives into products of biotechnological interest. This may facilitate, for example, the optimization of kinetic parameters and could circumvent current regulations regarding the release of genetically modified microorganisms into the environment. However, keeping individual proteins active and stable under field conditions will quite likely require considerable protein engineering.

Conclusion

As societies and local communities are increasingly concerned about mining activities and the enormous amount of toxic waste produced and released into the environment, the pressure is on to find novel methods to deal with these wastes. Cyanide-degrading microorganisms would enable bioremediation processes as eco-friendly alternatives to current chemical methods for treating cyanide-containing industrial residues and

polluted sites. Analysis of the alkaliphilic cyanotrophic bacterium *P. pseudoalcaligenes* CECT5344 generated the knowledge and a holistic picture of metabolic processes that allow this strain to survive and grow on cyanide. This information could be applied to develop synthetic biology strategies to optimize bioremediation processes. However, there are many different possible approaches, parameters and validations all of which require multidisciplinary research initiatives and development to further optimize Nature's ability to cope with and detoxify cyanide compounds.

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